GIV/Girdin Links Vascular Endothelial Growth Factor Signaling to Akt Survival Signaling in Podocytes Independent of Nephrin

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ABSTRACT
Podocytes are critically involved in the maintenance of the glomerular filtration barrier and are key targets of injury in many glomerular diseases. Chronic injury leads to progressive loss of podocytes, glomerulosclerosis, and renal failure. Thus, it is essential to maintain podocyte survival and avoid apoptosis after acute glomerular injury. In normal glomeruli, podocyte survival is mediated via nephrin-dependent Akt signaling. In several glomerular diseases, nephrin expression decreases and podocyte survival correlates with increased vascular endothelial growth factor (VEGF) signaling. How VEGF signaling contributes to podocyte survival and prevents apoptosis remains unknown. We show here that Ga–interacting, vesicle-associated protein (GIV)/girdin mediates VEGF receptor 2 (VEGFR2) signaling and compensates for nephrin loss. In puromycin aminonucleoside nephrosis (PAN), GIV expression increased, GIV was phosphorylated by VEGFR2, and p-GIV bound and activated Ga3 and enhanced downstream Akt2, mammalian target of rapamycin complex 1 (mTORC1), and mammalian target of rapamycin complex-2 (mTORC2) signaling. In GIV-depleted podocytes, VEGF-induced Akt activation was abolished, apoptosis was triggered, and cell migration was impaired. These effects were reversed by introducing GIV but not a GIV mutant that cannot activate Ga3. Our data indicate that after PAN injury, VEGF promotes podocyte survival by triggering assembly of an activated VEGFR2/GIV/Ga3 signaling complex and enhancing downstream PI3K/Akt survival signaling. Because of its important role in promoting podocyte survival, GIV may represent a novel target for therapeutic intervention in the nephrotic syndrome and other proteinuric diseases.


Podocytes are highly specialized glomerular epithelial cells uniquely organized into foot processes and filtration slits that are essential for glomerular filtration; podocyte injury often leads to foot process effacement and proteinuria.1–5 Podocyte injury is the initiating cause of many human and experimental glomerular diseases such as minimal change disease (MCD), FSGS, membranous nephropathy, and diabetic nephropathy.5–8 Podocytes have the capability to recover from mild injury; however, if injury is sustained, it can lead to their detachment from the glomerular basement membrane as well as cell death, followed by glomerulosclerosis and ESRD.1,5,7,8 Thus, it is essential to understand how podocytes respond to early injury and to characterize early signaling events that determine the reversibility of podocyte injury in order to develop novel therapeutics that can be used to...
Nephrin is currently assumed to act as the key signaling molecule that maintains the filtration slits and podocyte survival by recruitment of phosphoinositide 3-kinase (PI3K) and subsequent activation of prosurvival Akt signaling.3,9–11 In normal podocytes, nephrin interacts with podocin and CD2AP and recruits the p85 subunit of PI3K to initiate Akt activation and inhibit apoptosis in cultured podocytes.5,9,10,12 However, podocytes of nephrin-null mice have rates of apoptosis similar to wild-type mice.13 In addition, nephrin is downregulated and nephrin–p85 interaction12 and tyrosine phosphorylation of nephrin14 (required for activation of Akt) are diminished in patients in early stages of MCD.12,14 As well as in puromycin aminonucleoside nephrosis (PAN),15–17 a rat model of MCD, whereas podocytes survive.7,18,19 Furthermore, no significant differences in Akt phosphorylation and podocyte apoptosis were seen in nephrin-null and wild-type mice even though these mice showed foot process effacement, slit diaphragm disruption, and proteinuria.13 Thus, an alternative mechanism must be responsible for podocyte survival during the early, reversible stages of podocyte injury. Up to now, the signaling pathways that compensate for the nephrin-dependent survival pathway have remained unknown.

It has recently become evident that vascular endothelial growth factor receptor (VEGFR) 2 is the main receptor responsible for PI3K/Akt regulation and podocyte survival in response to autocrine vascular endothelial growth factor (VEGF)-A in cultured human and mouse podocytes.20 Akt functions as a critical regulator of growth factor–dependent survival and ameliorates cell apoptosis induced by toxic stimuli of a wide variety of cell types. There is growing evidence that VEGF signaling protects glomeruli from injury in numerous glomerular diseases, including MCD, FSGS, and diabetic nephropathy.21–24 In MCD, VEGF levels are upregulated in glomeruli from both normal and PAN rats. In glomeruli from both normal and PAN rats, GIV is expressed at the highest levels in podocytes (Figure 1D). It also codistributes with the intermediate filament protein vimentin, which is a specific marker for the primary processes of podocytes.25

**RESULTS**

**GIV Is Induced in Podocytes of PAN Rats**

PAN is a well established animal model to study reversible podocyte injury.26 Because GIV is known to enhance Akt survival signaling in other cells,28 we first assessed the expression of GIV in glomeruli isolated from normal and 7-day PAN rats. We found by quantitative immunoblotting that expression of GIV protein is increased approximately 4-fold (Figure 1, A and B) and by RT-PCR that GIV mRNA is increased 10-fold (Figure 1C) in 7-day PAN rats compared with normal controls, whereas Gαi3, a GIV effector, and Gβγ showed little change (Figure 1, A and B). By contrast, nephrin was decreased (42%) (Figure 1, A and B) as previously reported.16 These results indicate that both GIV transcript and GIV protein levels are significantly upregulated in PAN.

GIV binds VEGFR2 and mediates podocyte survival via activation of Gαi3 and enhancement of downstream PI3K/Akt/mammalian target of rapamycin (mTOR) signaling. Thus, as nephrin is downregulated, GIV takes over, prevents apoptosis, and maintains cell survival early after podocyte injury.

**Akt/mTOR Survival Signaling Is Maintained in PAN Rats**

In normal podocytes, survival signaling is mediated through the well defined nephrin/CD2AP-dependent, PI3K/Akt pathway,8 but how cell survival is maintained after podocyte injury when nephrin expression decreases7,18,19 has remained unknown. Because we found GIV to be upregulated in PAN and GIV is known to enhance Akt signaling in other cell types,28 we anticipated that Akt signaling might be maintained or increased in nephritic glomeruli. At 7 days after puromycin aminonucleoside (PA) injury, activation of Akt as determined by its phosphorylation at S473 was increased (2.2-fold) (Figure 2A) even though nephrin was decreased (Figure 1, A and B). Similarly, when in vitro differentiated mouse podocytes were treated with PA (30 μg/ml), there was very little caspase 3 cleavage (5%) in podocytes at early time points (24 hours) (Figure 2, C and D), indicating that very little apoptosis occurs. However, when treatment was extended to 48 or 72 hours, which mimics prolonged injury in PAN rats, cleavage of caspase 3 increased (28% and 43%, respectively) (Figure 2, C and D).
It was recently reported that Akt2 is the main Akt isoform expressed in mouse and human podocytes and that Akt2 activation protects podocytes during CKD. We confirmed that Akt2 is the major Akt isoform expressed in rat glomeruli and differentiated mouse podocytes (Figure 2E) and found that phosphorylation of Akt2 at S473 is greatly increased in PAN glomeruli (Figure 2F). Because mTOR complex 2 (mTORC2) is known to phosphorylate Akt at S473 upon growth factor stimulation, this finding suggested that Akt2 might be activated by mTORC2 in PAN. To find out whether this is the case, we examined activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1), a specific substrate of mTORC2 kinase, and found that pSGK1 was enhanced 2.6-fold in PAN glomeruli (Figure 2, G and H). mTOR complex 1 (mTORC1) is also activated by growth factors downstream of PI3K/Akt so we checked two substrates of mTORC1, S6 kinase (S6K) and S6 ribosomal protein (S6RP), and found that their activation is increased 2-fold in glomeruli from PAN rats (Supplemental Figure 1). These results indicate that in the absence of nephrin, downstream mTORC2-Akt2 survival signaling and mTORC1 cell growth signaling are maintained in podocytes via nephrin-independent pathways.

Interaction of GIV with Activated VEGFR2 Is Increased in PAN Rats

Because GIV is known to bind to activated RTKs, including VEGFR, and expression of GIV as well as VEGF-A and its receptors is increased in PAN, we reasoned that GIV binding to VEGFR2 might be increased in PAN. Interestingly, we found that expression of VEGF-A and total VEGFR2 was increased approximately 2.5-fold in glomeruli from 7-day PAN rats (Figure 3A), and activation of VEGFR2 (p-VEGFR2) increased 5.2-fold in both PAN glomeruli (Figure 3A) and PA-treated podocytes (Figure 3B). Moreover, the amount of GIV that coimmunoprecipitated with VEGFR2 from glomerular lysates of PAN rats was increased 3.5-fold (Figure 3C). Thus, VEGF signaling and binding of GIV to VEGFR2 are enhanced in podocytes after PA injury.

GIV Is Phosphorylated at Ser1416 and Tyr1764 in PAN

It was recently shown that, upon binding to an RTK, GIV is phosphorylated at Tyr1764 and Tyr1798, and that tyrosine phosphorylated GIV directly binds and activates class I PI3Ks, thereby enhancing Akt phosphorylation and actin remodeling during cell migration. In addition to enhancing Akt signaling, GIV is also a substrate for Akt, and phosphorylation of GIV at Ser1416 by Akt promotes binding of GIV to actin and actin remodeling. Together these phosphorylation events indicate that GIV-dependent signaling is “on.” To check whether PAN injury turns on GIV-dependent signaling, we immunoblotted...
glomerular lysates with antibodies that specifically recognize pTyr1764 or pSer1416 of GIV. Phosphorylation of GIV at both of these sites was significantly increased in PAN glomeruli (Figure 3D), indicating that GIV is activated and that GIV-dependent signaling is specifically induced upon PAN injury.

**GIV Binding to Goi3 and Activation of Goi3 Are Increased in PAN**
We established earlier that after growth factor stimulation of HeLa cells, GIV binds inactive Goi3 and serves as a GEF that activates Goi3, thereby releasing Gβγ subunits that directly bind PI3K and activate PI3K/Akt signaling.21 To
investigate whether G\(\alpha\)i3 and GIV interact in glomeruli, we immunoprecipitated endogenous G\(\alpha\)i3 and immunoblotted for GIV. The amount of GIV that coimmunoprecipitated with G\(\alpha\)i3 was greatly increased (4.2-fold) in PAN glomeruli, indicating that interaction between G\(\alpha\)i3 and GIV is enhanced (Figure 4A). To determine whether binding is state dependent, we carried out in vitro binding assays with recombinant glutathione S-transferase (GST)-G\(\alpha\)i3 in the presence of GDP and AlF\(_4^-\) (active state).44 We found that GIV from PAN glomeruli bound inactive but not active G\(\alpha\)i3, whereas GIV from normal glomeruli did not bind either active or inactive G\(\alpha\)i3 (Figure 4B). Similarly, interaction between inactive GST-G\(\alpha\)i3 and GIV was increased in PA-treated podocytes (data not shown). Next we checked whether PA injury leads to activation of G\(\alpha\)i3 using an antibody that recognizes only active G\(\alpha\)i3 (G\(\alpha\)i3--GTP)\(^45\) and found that the ratio of active/total G\(\alpha\)i3 was increased (1.7-fold) in PAN glomeruli (Figure 4C and D).

We recently found that the ability of GIV to activate G\(\alpha\)i3 is regulated by phosphorylation at S1689, as GIV-pS1689 fails to bind and activate G\(\alpha\)i3.\(^46\) Because interaction between GIV and G\(\alpha\)i3 is increased in PAN, we reasoned that the level of phosphorylation of GIV at S1689 might be altered. When we adjusted the amount of glomerular lysate so that the level of GIV was equal in both normal and PAN glomeruli, phosphorylation of GIV at S1689 was >2-fold higher in normal glomeruli (Figure 4, E and F). These results suggest that binding and activation of GIV by G\(\alpha\)i3 is inhibited by phosphorylation at S1689 in normal glomeruli which prevents its interaction with G\(\alpha\)i3,\(^46\) whereas phosphorylation of GIV at S1689 is reduced after PA injury which allows it to bind and activate G\(\alpha\)i3.

**GIV Mediates Assembly of a VEGFR2/ G\(\alpha\)i3 Molecular Complex**

Our findings that VEGFR2 and Akt are strongly activated and that GIV binds activated VEGFR2 as well as G\(\alpha\)i3 in glomeruli from nephrotic rats suggest that GIV provides an important link between VEGF-A stimulation and Akt survival signaling in podocytes. Next we asked whether GIV enhances Akt signaling through formation of a VEGFR2/GIV/G\(\alpha\)i3 complex in podocytes in response to VEGF-A. To determine whether this is the case, cultured podocytes were treated with VEGF-A for 30 minutes, followed by immunoprecipitation with anti-VEGFR2 IgG and immunoblotting for G\(\alpha\)i3, VEGFR2, and GIV. We found that little, if any, G\(\alpha\)i3 coprecipitated with VEGFR2 from lysates of starved cells, but both GIV and G\(\alpha\)i3 coprecipitated with VEGFR2 after VEGF stimulation (Figure 5A). Similar experiments on lysates from control versus PA-treated podocytes showed that the interaction of VEGFR2 with GIV and G\(\alpha\)i3 is also greatly increased after PA treatment (Figure 5B). Thus, both VEGF activation and PA treatment trigger the assembly of VEGFR2/GIV/G\(\alpha\)i3 protein complexes in podocytes.

**GIV Is Required for Actin Remodeling, Cell Migration, Akt2/mTOR activation, and Cell Survival in Cultured Podocytes**

GIV has been shown to act upstream of Akt and to be essential for Akt activation, cell survival, actin remodeling, and cell migration in several cell types (i.e., epithelial cells,\(^29\) macrophages,\(^47\) and endothelial cells\(^42\)). Our results in PAN rats suggest that the same is true for podocytes. To directly test whether this is the case, we depleted GIV from differentiated cultured mouse podocytes by the peptide transducing domain-double stranded RNA binding domain (PTD-DRBD) method,\(^48\) which is an effective way to introduce small interfering RNAs (siRNAs) into these cells (Figure 6A). In GIV-depleted podocytes, Akt phosphorylation was attenuated (Figure 6, A and B), the actin cytoskeleton reorganized (Figure 6C), and cell migration was impaired (Figure 6, D and E). Moreover, GIV-depleted cells showed increased Bax...
(1.8×), a proapoptotic marker, and decreased antiapoptotic markers Bcl-2 and Bcl-XL (75% and 54%, respectively) (Figure 6F). GIV depletion also abolished phosphorylation of Akt and SGK1 in response to VEGF-A stimulation (Figure 6, G and H). Thus, GIV plays a key role in enhancing prosurvival Akt signaling and actin remodeling after growth factor stimulation in cultured podocytes.

**The GEF Function of GIV Is Required for Podocyte Survival**

Next we asked whether activation of Gai3 by GIV is required for podocyte survival. siRNA resistant GIV wild-type (GIV-WT) or a specific mutant, GIV F1685A (GIV-FA), which fails to bind and activate Gai3, were expressed in podocytes using adenoviral vectors. In cells expressing GIV-WT, Akt phosphorylation (S473) in response to VEGF-A stimulation (Figure 7, A and B) and expression of antiapoptotic markers (Figure 7C) were higher than in those expressing GIV-FA. Notably, expression of GIV-WT rescued cell viability in GIV-depleted cells as shown by decreased caspase 3 cleavage, whereas expression of GIV-FA did not because caspase cleavage...
of podocyte lysates from untreated and PA-treated (24-hour) podocytes. IB, immunoblot; IP, immunoprecipitate.

**DISCUSSION**

It has recently become evident that both VEGF signaling and Akt activation function in maintaining cell viability after podocyte injury. The molecular pathways that connect growth factor signaling and downstream Akt survival signaling after podocyte injury have remained unknown. Our data clearly point to GIV as the critical linker between upstream VEGFR and downstream PI3K/Akt survival signaling in PAN, a model of MCD. We found that during the early phases of PA injury, VEGFR2 is activated and GIV is upregulated. GIV assembles a VEGFR/GIV/Gαi3 signaling complex that promotes podocyte survival through activation of Gαi3 and enhancement of PI3K/Akt downstream signaling (see Figure 8B). In keeping with previous findings on EGFR, our results suggest that tyrosine phosphorylation of GIV by VEGFR2 is the trigger for assembly of the VEGFR2/GIV/Gαi3 complex and subsequent activation of Gαi3. The essential role of GIV in this scenario is illustrated by our findings that in the absence of GIV, Akt signaling is reduced and podocytes undergo apoptosis. The crucial role of GIV’s GEF function is illustrated by the fact that these effects can be reversed by introducing human GIV wild-type protein but not a GIV mutant (GIV-FA) that is incapable of binding and activating Gαi3. Thus, after acute injury as nephrin levels decrease GIV takes over the task of enhancing Akt survival signaling in response to VEGF, and activation of Gαi3 is required for this effect.

We show here that Akt2 is the isoform that is highly expressed in rat glomeruli and is activated in PAN, and that GIV acts upstream of Akt2, mTORC1, and mTORC2. Both mTORC1 and mTORC2 are activated early after PAN injury as indicated by increased phosphorylation of their respective substrates, S6K and SGK1. These results are in keeping with previous studies on podocyte-specific, mTOR-deficient mice, which established that both mTORC1 and mTORC2 are essential for the integrity and proper functioning of the glomerular filtration barrier.

**GIV Coordinates Multiple Signaling Pathways**

We show here that GIV coordinates growth factor, G protein, and PI3K/Akt/mTOR signaling to promote cell survival and prevent apoptosis in podocytes after PAN injury. GIV is uniquely organized to directly receive incoming receptor signals and to modulate them via G proteins and was previously shown to regulate such diverse cell processes as cell migration, cancer metastasis, and autophagy. The C terminus serves as a common platform for binding RTKs, G proteins, and Akt. Interactions of GIV with its binding partners and resultant downstream signaling are controlled by specific phosphorylation events: Phosphorylation of GIV at Tyr1764 and Tyr1798 by RTKs regulates GIV’s ability to activate PI3Ks and activate Akt, and phosphorylation of GIV at S1689 prevents binding of GIV to Gαi3. We show here that in PAN rats in response to stress, activation of VEGFR2 is increased, phosphorylation of GIV at Tyr1764 is enhanced, and phosphorylation at S1689 is suppressed thus promoting assembly of VEGFR2/GIV/Gαi3 complexes, facilitating activation of Gαi3, and enhancing downstream PI3K/Akt/mTOR survival signaling. The PAN model we used is reversible;

**Figure 5.** GIV binds VEGFR2 and mediates assembly of a VEGFR2/GIV/Gαi3 molecular complex in both VEGF-treated and PA-treated podocytes. (A) GIV and Gαi3 coimmunoprecipitate with VEGFR2 in VEGF-stimulated but not in serum-starved podocytes. Differentiated podocytes are serum starved or stimulated with VEGF (40 ng/ml) for 30 minutes, and cell lysates (500 μg) are incubated with anti-VEGFR2 IgG. Control IgG is incubated with a mixture of lysates from starved and VEGF-stimulated podocytes. Immunoprecipitates and cell lysates (50 μg) are immunoblotted for GIV, VEGFR2, p-Akt (S473), t-Akt, Gαi3, and β-actin. Control IgG is incubated with a mixture of podocyte lysates from untreated and PA-treated (24-hour) podocytes. IB, immunoblot; IP, immunoprecipitate.

(B) Kinetics of assembly of both Gαi3 and GIV with VEGFR2 is greatly enhanced after PA treatment (24 hours) of differentiated podocytes, indicating that their interaction is greatly increased. Differentiated podocytes are incubated (24 hours) with or without PA (30 μg/ml), and cell lysates (500 μg) are incubated with anti-VEGFR2 or control IgG. Immunoprecipitates, and cell lysates (50 μg) are analyzed by immunoblotting with antibodies against GIV, VEGFR2, Gαi3, and β-actin. Control IgG is incubated with a mixture of podocyte lysates from untreated and PA-treated (24-hour) podocytes. IB, immunoblot; IP, immunoprecipitate.

was increased 3-fold compared with GIV-WT rescued podocytes (Figure 7D). These results indicate that the GEF function of GIV, and by implication activation of Gαi3, is required for maintaining cell survival and antiapoptotic signaling in podocytes.

Taken together, our results support that at early stages of podocyte injury induced by PA treatment, GIV turns on Gαi3 through its GEF function and provides a critical link between upstream VEGFR2 signaling and downstream Akt2/mTOR survival signaling (Figure 8).
Figure 6. GIV enhances Akt phosphorylation, actin remodeling, cell migration, and survival in podocytes. (A) p-Akt is decreased in GIV-depleted podocytes. Differentiated podocytes are transfected with control or GIV siRNA. Forty-eight hours later, cell lysates are immunoblotted for GIV, p-Akt (S473), t-Akt, and β-actin. (B) Quantification of data from 10 experiments expressed as the fold change in GIV siRNA versus Ctrl siRNA-treated podocytes (normalized to β-actin) (**P<0.001; mean±SD). (C) Podocytes treated with control siRNA show stress fibers (arrows), whereas GIV-depleted podocytes show prominent cortical actin (arrowheads). Control or GIV siRNA-treated podocytes are fixed and stained with Phalloidin Alexa Fluor 594 and DAPI. (D) Cell migration assay. Twenty-four hours after wounding, podocytes treated with control siRNA cover most of the wound area, whereas closure is impaired in GIV-depleted cells (GIV siRNA). Podocyte monolayers are scratch-wounded and examined by phase-contrast microscopy after 0 or 24 hours. (E) Quantification of data from eight experiments as in D (**P<0.001; mean±SEM). (F) After siRNA depletion of GIV (75%), proapoptotic Bax is increased (1.8-fold) and antiapoptotic Bcl-2 and Bcl-XL are decreased (to 25% and 46%, respectively) compared with controls (Ctrl siRNA). mRNA levels are determined by quantitative real-time PCR of total RNA (normalized to β-actin mRNA) (*P<0.05; **P<0.001; mean±SEM n=5). (G) VEGF-mediated phosphorylation of Akt and SGK1 is abolished in GIV-depleted podocytes. Podocytes are transfected with control or GIV siRNA by the PTD-DRBD method. Forty-eight hours after transfection, cells are serum starved or stimulated with VEGF (40 ng/ml) for 30 minutes, and cell lysates are immunoblotted for p-Akt (S473), p-SGK1 (S422), and β-actin. (H) Quantification of data from three representative experiments as in G expressed as the fold change in VEGF-mediated stimulation of p-Akt and p-SGK1 (normalized to β-actin) compared with starved controls (**P<0.001; mean±SD). DAPI, 4,6-diamidino-2-phenylindole; IB, immunoblot; Ctrl, control. Bar, 10 μm.
thus, the enhanced VEGF stimulation, GIV activation, and Akt signaling stimulated by PA treatment must eventually subside. Because GIV activation is controlled by tyrosine phosphorylation at two specific sites, we anticipate that the GIV/PI3K/Akt signaling pathway might be terminated by specific phosphatases such as Src homology domain 2-containing protein-tyrosine phosphatase-1, which binds and dephosphorylates GIV,\(^51\) and by phosphorylation of GIV at S1689 by PKC\(\mu\), which inhibits G\(\alpha\)i3-GIV coupling.\(^46\)

GIV Mediates Actin Remodeling in Podocytes

The normal podocyte has an elaborate actin cytoskeleton with a prominent actin network in the cell body and foot processes that maintains the characteristic foot process and filtration slit organization.\(^1\)\(^-\)\(^5\)\(^-\)\(^3\)\(^2\)\(^5\) It is assumed that in the normal glomerulus, the nephrin/VEGFR2 signaling complex regulates the actin cytoskeleton and maintains foot process architecture.\(^52\) The actin network undergoes dramatic reorganization in diseases that are associated with foot process effacement and proteinuria (e.g., PAN).\(^3\)\(^-\)\(^5\)\(^3\)\(^2\)\(^5\) Previous work established that GIV binds Akt and is phosphorylated at S1416 by Akt, which mediates actin remodeling during cell migration in other cell types.\(^42\) GIV has also been shown to be involved in VEGF-dependent actin reorganization and cell migration during VEGF-mediated angiogenesis in endothelial cells.\(^54\) Our finding that GIV is phosphorylated at S1416 by activated Akt

\(\text{Figure 7.}\) The GEF function of GIV is required for podocyte survival. (A) VEGF-mediated prosurvival Akt signaling is enhanced in podocytes overexpressing GIV-WT (WT) and is inhibited in podocytes overexpressing GIV-FA (FA). Podocytes are transduced with Ad-GIV-WT (WT) or Ad-GIV-FA (FA), or control adenovirus (AdC). Forty-eight hours later, they are switched to 0.4% serum for 24 hours and then stimulated with VEGF (40 ng/ml) for 30 minutes. Equal amounts of cell lysate are immunoblotted for GIV, p-Akt (S473), and \(\beta\)-actin. (B) Quantification of data in A (normalized to \(\beta\)-actin). Data are expressed as the fold change in AdC, Ad-GIV-WT, or Ad-GIV-FA–treated podocytes. (C) Depletion of endogenous GIV in cells transduced with control virus (AdC) decreases expression of the antianoptotic markers Bcl-2 (30%) and Bcl-XL (25%). Overexpression of GIV-WT prevents this reduction in expression of Bcl-2 and Bcl-XL, whereas overexpression of GIV-FA does not. Podocytes are transduced with control (AdC), Ad-GIV-WT, or Ad-GIV-FA, followed (24 hours later) by control or GIV siRNA. After 48 hours, equal amounts of cell lysates are immunoblotted for GIV or \(\beta\)-actin. RNA is isolated and relative mRNA levels of Bcl-2 and Bcl-XL are determined by quantitative real-time PCR. (D) Addition of PA (36 hours) to GIV siRNA-treated podocytes transduced with control virus (lane 6) leads to increased caspase 3 cleavage (1.7-fold) compared with control siRNA-treated cells (lane 5). Overexpression GIV-WT (lane 7) inhibits the PA-mediated increase in caspase 3 cleavage but overexpression of GIV-FA (lane 8) does not. No caspase cleavage is seen in untreated cells (lanes 1–4). Podocytes are transduced with control virus (AdC), Ad-GIV-WT, or Ad-GIV-FA, followed (24 hours later) by control or GIV siRNA. After 48 hours, they are treated with 30 \(\mu\)g/ml PA for 36 hours. Equal amounts of cell lysates are immunoblotted for GIV, caspase 3, and \(\beta\)-actin. (E) Quantification of data in D (normalized to \(\beta\)-actin). Data are expressed as the percentage of active caspase 3/total caspase 3 (procaspase 3+active caspase 3) (**P<0.001; mean±SD n=3). IB, immunoblotting.
in PAN rats, which promotes its binding to and remodeling of actin in response to VEGF/VEGFR2 activation,\(^{42}\) suggests that GIV plays a role in enhancing actin remodeling in podocytes during foot process effacement in PAN.

**Relevance of GIV Findings in the PAN Rat Model to Human Glomerular Diseases**

Our results demonstrate that VEGF-A and VEGFR2 are upregulated and autophosphorylated of VEGF/VEGFR2 is increased in glomeruli from PAN rats. This is in keeping with previous studies demonstrating that VEGF/VEGFR2 coupling is prominent in podocytes of biopsies from patients with MCD,\(^{55}\) crescentic nephritis,\(^{23}\) lupus nephritis,\(^{23}\) IgA and membranous nephropathy,\(^{23}\) diabetic nephropathy,\(^{22}\) and HIV-associated nephropathy.\(^{26}\) VEGF-A is abundantly expressed and secreted by podocytes and has been suggested to protect renal glomeruli from injury\(^{21,22}\) and to activate the PI3K/Akt survival pathway and protect glomerular endothelial cells,\(^{57}\) and tubular epithelial cells\(^{58}\) as well as podocytes\(^{59}\) from apoptosis. It seems likely that all of these events are mediated through GIV. Moreover, activation of Akt has been shown to be essential for maintaining podocyte viability in stress induced by nephron reduction\(^{56}\) and oxidized LDL-mediated injury.\(^{56}\) GIV’s ability to amplify Akt signaling is not restricted to a single receptor or class of receptor: It has been shown to serve as a common platform that coordinates signaling downstream of multiple growth factor receptors (EGFR, IGF1R, InsR) as well as some G protein–coupled receptors.\(^{28}\) On the basis of our findings and the properties of GIV, we predict that GIV might similarly act as a linker between other RTKs (e.g., insulinR\(^ {60}\)) and downstream Akt/mTOR signaling to promote podocyte survival early in the course of other podocytopathies.

**CONCISE METHODS**

**Reagents and Antibodies**

All reagents were of analytical grade and were obtained from Sigma-Aldrich or Fisher Biotech; cell culture media were purchased from Invitrogen. PA, type I collagen, and isopropyl β-D-thiogalactopyranoside were from Sigma-Aldrich, recombinant mouse VEGF was from R&D Systems, and mouse γ-IFN was from BD Pharmingen. A rabbit polyclonal antibody (pAb), GIV (CT3347), against the C terminus of human GIV (amino acids 1574–1843) was raised and affinity-purified as previously described\(^ {61}\) and used for staining full-length GIV in kidney sections by immunofluorescence. Rabbit IgG against the extracellular domain of rat nephrin was raised and purified as previously described.\(^ {62}\) A mouse mAb that recognizes the ectodomain of rat podocalyxin (5A) was previously described.\(^ {63}\) Rabbit pAbs for GIV/ Girdin (T-13), pan-GBP (M-14), Gα3 (C-10), phospho–VEGFR2/ phospho-Flk-1(Y996), p-SGK1 (S422), a mouse mAb for VEGFR2/Flk-1 (A-3), control rabbit IgG, and control mouse IgG were purchased from Santa Cruz Biotechnology. Rabbit pAbs against phospho-Akt (S473), Akt1, Akt2, p-S6K (T389), p-S6RP (S235/236), and caspase 3, and mouse mAbs against Akt3 and total Akt were purchased from Cell Signaling Technology. pAbs were also from Cell Signaling Technology. A mouse mAb for VEGF was from BD Pharmingen and those against vimentin, SGK1, and β-actin were from Sigma-Aldrich. Affinity-purified rabbit pAbs for phospho-GIV (Y1764) and phospho– GIV (S1689) were from Roche and those for phospho–GIV (S1416) were from Immuno-Biologic Laboratories. A mouse mAb specific for activated Gαi subunits was kindly provided by Dr. Graeme Milligan; IRDye 800CW goat anti-mouse IgG (H+L) used for immunoblotting were from Li-Cor Biosciences. For immunofluorescence, 4,6-diamidino-2-phenylindole, Alexa Fluor 594 phalloidin, highly cross-adsorbed Alexa Fluor 594 goat anti-mouse IgG F(ab’)2, and Alexa Fluor 488 goat anti-rabbit IgG F(ab’)2 were purchased from Invitrogen.

**Induction of PAN Nephrosis**

Male rats (approximately 150 g; Charles River Laboratories) were injected once intraperitoneally with PA (15 mg/100 g body wt) as
previously described. Animals were euthanized on day 7 after injection. All animal experiments were done according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and the experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the University of California, San Diego.

**Culture and Transfection of Mouse Podocytes**

Conditionally immortalized mouse podocytes (a gift from Dr. Peter Mundel) were cultured at 33°C in RPMI 1640 (Invitrogen) containing 10% FBS (HyClone), 100 U/ml of penicillin–streptomycin, and 10 U/ml of mouse γ-IFN. To induce differentiation, podocytes were replated and cultured at 37°C in plates coated with 10 mg/ml type I collagen in the absence of γ-IFN. After 14-day differentiation, siRNA depletion was performed by using the PTD-DRBD mediated delivery method. PTD-DRBD was purified from *Escherichia coli* and mixed with siRNA. Silencer negative control siRNA was purchased from Ambion. siRNA for targeting mouse GIV (sense: 5'-GCCAAAGCUUACCUGAUAAUTT-3' and anti-sense: 5'-AUUGAGGUACGCUUGCGCTT-3') was designed and synthesized by Allele Biotechnology & Pharmaceuticals Inc. Cells were harvested 48 hours after transfection and used for immunoblotting, immunoprecipitation, or immunofluorescence.

**Preparation of Lysates and Immunoblotting**

Rat glomeruli were isolated from kidney cortices of male Sprague-Dawley rats by grading sieving as previously described. Isolated glomeruli or cultured podocytes were lysed in RIPA buffer (100 mM sodium phosphate, 150 mM NaCl, 2 mM EDTA, 2 mM dithiothreitol [DTT], 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, phosphatase inhibitor cocktail III [Sigma-Aldrich], and protease inhibitor cocktail [Roche Diagnostics], 0.5 mM sodium orthovanadate, 10 mM NaF, 10 mM sodium polyphosphate). The protein concentration of glomeruli or cultured podocyte lysates was measured with a BCA Protein Assay Kit (Pierce Biotechnology Inc.) according to the manufacturer’s instructions. Glomeruli or cultured podocyte lysates were mixed with an equal volume of 5X Laemmli SDS sample buffer and boiled for 10 minutes. Proteins were separated on 8% or 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes (EMD Millipore) as described. Membranes were blocked in blocking buffer (5% BSA, 0.1% Tween-20 in PBS) and incubated with primary antibodies (4°C overnight) and then with secondary antibodies (1 hour at room temperature). Bands were scanned and quantified by two-color detection with the Odyssey Infrared Imaging system (Li-Cor Biosciences). Primary antibody dilutions were as follows: 1:500 for GIV, VEGFR2, p-VEGFR2 (Y996), nephrin, p-Akt (S473), p-SGK (S422), SGK, p-S6K (T389), p-S6RP (s235/236), GRII, G3b, caspase 3, VEGF-A, and p-GIV (S1689); 1:250 for p-GIV (S1416) and p-GIV (Y1764); and 1:1000 for t-Akt and β-actin. Secondary antibody dilutions were 1:15,000 for IRDye 680 goat anti-rabbit and 1:15,000 for IRDye 800 goat anti-mouse IgG.

**Immunofluorescence Microscopy**

For imaging of semithin sections of glomeruli, rat kidney samples were immersion fixed in 4% paraformaldehyde for 4 hours at 4°C, cryoprotected, and frozen in liquid nitrogen. Semithin cryosections (0.5 μm) were cut with a Leica Ultracut UCT microtome equipped with an FCS cryoattachment at −100°C. Sections were blocked for 1 hour in PBS containing 5% normal goat serum, 2% fish skin gelatin, and 0.1% acetylated BSA, incubated with primary antibodies 2 hours at room temperature (GIV-CT3347 pAb, 1:100; vimentin mAb, 1:1000; and podocalyxin mAb 5A, 1:1000), followed by detection with Alexa 594 goat anti-mouse and Alexa 488 goat anti-rabbit IgG in blocking buffer for 1 hour. Cultured mouse podocytes were fixed at room temperature with 3% paraformaldehyde for 30 minutes, permeabilized (0.1% Triton X-100) for 10 minutes, and incubated for 1 hour each with primary and secondary antibodies as previously described. Primary antibody dilutions were as follows: 1:10 for GIV-CT antibody and 1:100 for Podocalyxin (5A). Secondary antibody dilutions were 1:1000 for Alexa 594 goat anti-mouse and Alexa 488 goat anti-rabbit IgG, 1:1000 for Alexa Fluor 594 phalloidin, and 1:3000 for 4,6-diamidino-2-phenylindole (Invitrogen). Samples were examined with a Zeiss Axioshot microscope (Carl Zeiss Inc., Thornwood, NY), and images were acquired using Velocity software (Improvision) and processed using ImageJ (NIH) and Photoshop software (Adobe Systems).

**Protein Purification**

GST or GST-Gri3 fusion constructs were expressed in *E. coli* strain BL21 (DE3) (Invitrogen) as previously described and induced overnight at 25°C with 1 mM isopropyl β-D-1-thiogalactopyranoside (Sigma-Aldrich). Pelleted bacteria from 1 liter of culture media were resuspended in 10 ml of GST lysis buffer (25 mM Tris-HCl, pH 7.5, 20 mM NaCl, 1 mM EDTA, 20% glycerol, 1% Triton X-100, 2× protease inhibitor cocktail [Roche Diagnostics]). After sonication, lysates were centrifuged at 4°C (12,000×g for 20 minutes). Solubilized proteins were affinity-purified on glutathione-Sepharose 4B beads (GE Healthcare). Proteins were eluted, dialyzed overnight against PBS, and stored at −80°C.

**Immunoprecipitation and In Vitro Binding Assays**

Lysates for immunoprecipitation or in vitro binding assays were prepared by resuspension of glomeruli or podocytes in Triton X-100 lysis buffer (25 mM HEPES, 125 mM K-acetate, 5 mM Mg-acetate, 0.4% Triton X-100, phosphatase inhibitor cocktail III [Sigma-Aldrich], and protease inhibitor cocktail [Roche Diagnostics], 400 mM Na orthovanadate, pH 7.2) and passed through a 28-gauge needle at 4°C and cleared (14,000×g for 10 minutes) before use in subsequent experiments. For immunoprecipitation, rat glomerular lysates (1 mg total protein) or mouse podocyte lysates (500 μg total protein) were incubated 3 hours or overnight at 4°C with 2 μl anti-VEGFR2 or anti-Gri3 or rabbit IgG (Santa Cruz Biotechnology) in Triton X-100 lysis buffer. Protein A magnetic beads (EMD Millipore) were added and incubated at 4°C for an additional 1 hour. Beads were washed (×4) with 1 ml of PBS-T (4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20, 10 mM MgCl2, 5 mM EDTA, and 2 mM DTT), and the bound immune complexes were eluted by boiling in 2× Laemmli SDS sample buffer. For in vitro protein binding assays, purified GST-Gri3 fusion proteins or GST alone (10 μg) were immobilized on glutathione-Sepharose beads and incubated in...
binding buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.4% NP-40, 10 mM MgCl$_2$, 5 mM EDTA, 2 mM DTT, protease inhibitor cocktail [Roche Diagnostics]) containing either 30 μM GDP or 30 μM GTP, 30 μM AlCl$_3$, and 10 mM NaF for 90 minutes at room temperature. We added 500 μg of glomerular or mouse podocyte lysate in Triton X-100 lysis buffer to each tube and binding reactions were carried out overnight at 4°C with constant rotation. Beads were washed four times with 1 ml PBS-T wash buffer supplemented with GDP or GTP, AlCl$_3$, and NaF, and boiled in 2 X Laemml SDS sample buffer.34

Cell Migration Assays
Differentially wild-type and GIV-depleted podocytes were plated on type I collagen–coated coverslips in six-well plates until 100% confluent. Each coverslip was then scratched with a sterile 200-μl pipette tip, washed with PBS, and placed into fresh medium. Images were captured by phase-contrast microscopy under a ×10 objective at 0 and 24 hours after wounding and analyzed using ImageJ software to quantify cell migration (expressed as the percentage of wound area covered) as previously described.47,67

Semiquantitative RT-PCR
Total RNA was extracted with the RNeasy Protect Mini Kit (Qiagen) from rat glomeruli according to the manufacturer’s instructions. cDNA was prepared using a SuperScript III RT kit (Invitrogen). The sequence-specific primers for GIV with a size of 204 bp were forward primer 5’-TAT GGC ACT TTA CCT GGT GCA A-3’ and reverse primer 5’-CCT AGA CCT GCT TTT TGA ATT TCT-3’. The sequence-specific primers for glyceraldehyde-3-phosphate dehydrogenase with a size of 555 bp were forward primer 5’-AAT GCA TCC TGC ACC ACC AAC TGC-3’ and reverse primer 5’-GGA GGC CAT GTA GCC CAT GAG GTC-3’. The PCR conditions were as follows: 5 minutes at 94°C for the initial denaturation, followed by 1 minute at 94°C, 1 minute at 50°C, and 1 minute at 72°C for amplification, and a final extension at 72°C for 5 minutes. The PCR products were separated on a 1.5% agarose gel, and an image of the gel stained with ethidium bromide was captured using the Quantity One (Bio-Rad). Data were expressed as the mean±SD or mean±SEM. Statistical significance was evaluated using the t test. P<0.05 and P<0.001 were considered statistically significant.

Adenoviral Vectors
Hemagglutinin-tagged full-length human GIV (wild-type or F1685A) resistant to siRNA against murine GIV was cloned into pShuttle-cytomegalovirus vector.68 The resultant plasmid was linearized with PacI and transfected into Cre8 cells (a derivative of HEK293T cells, a gift from Dr. Ora Weisz, University of Pittsburgh). Serial amplification of the adenovirus and subsequent purification of the adenoviral particles on cesium chloride gradients was carried out as previously described.68 Purified viral particles were dialyzed against PBS, aliquoted, and stored at −80°C. For expression of WT-GIV and GIV-F1685A, cultured cells were incubated with the respective viruses at 50 multiplicity of infection for 5 hours. The medium was replaced and cultures were maintained for an additional 3–5 days before being used for protein or functional assays.

Quantitative Real-Time PCR
Quantitative real-time PCR was carried out on an ABI Prism 7300 Sequence Detection System (Applied Biosystems) using SYBR GreenER qPCR SuperMix for ABI PRISM (Invitrogen) according to the manufacturer’s instructions. The previously verified primers for mouse genes were as follows: Bcl-2 forward primer 5’-AGGAGCAAGGTGCTCAACAAGA-3’ and reverse primer 5’-GCAATTTCCACACTGCTCT-3’,69 Bcl-xL forward primer 5’-GCTGGACACCTTTTTGGAGAT-3’ and reverse primer 5’-GTCTGGTACCTCCTCGACTG-3’,70 β-actin forward primer 5’-AGATGTTGACAGAAGCAG-3’70 and reverse primer 5’-GGCGAAGTTAGGGTTTGTCA-3’,70 and Bax forward primer 5’-TGCAGAGGATATTTGCTGAC-3’ and reverse primer 5’-GATCGTCTGCCGACCTTAG-3’.69 Specific primers for mouse GIV were designed by online software as follows: forward primer 5’-GTGATCTCTACTGCTGAAGG-3' and reverse primer 5’-TGTTGTCCCTAGACCTGCT-3’. PCR reactions were carried out at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. All reactions were run in triplicate. After data collection, the relative mRNA expression level of a specific gene in the total RNA was calculated and normalized using mouse β-actin as an internal control.

Statistical Analyses
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DISCLOSURES
None.

REFERENCES


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